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PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS

This is a Divisional application of prior application Serial No. 08/087,471, filed on July 2, 1993, now U.S. Patent No 5,429,242 which is a File Wrapper Continuation of Serial No. 07/821,478, filed on January 15, 1992, now abandoned which is a Continuation of Serial No. 07/404,970, filed on September 8, 1989, now abandoned, which is a Continuation-In-Part of Serial No. 07/393,851, filed on August 14, 1989, which issued as U.S. Patent No. 5,010,010 on April 23, 1991, which application, in turn, is a File Wrapper Continuation of Serial No. 06/921,684, filed on October 22, 1986, abandoned.

FIELD OF THE INVENTION

This invention relates to genetically engineered microorganisms containing DNA coding for human preproparathyroid hormone.

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of Application Serial No. 07/393,851 filed August 14, 1989, which is a continuation of Application Serial No. 06/921,684 filed October 22, 1986, now abandoned.

A number of proteins and peptides that are normally synthesized by mammalian cells have proven to have medical, agricultural and industrial utility. These proteins and peptides may be of different molecular size and have a number of different functions, for example, they may be enzymes, structural proteins, growth factors and hormones. In essence both prot ins and peptides are composed of linear sequences of amino acids which form secondary and tertiary structures that are necessary to convey the biological activity. Human parathyroid hormone has a relatively small molecular weight, which has made it possible to synthesize the peptide chemically by the sequential addition of amino acids. Thus, parathyroid hormone is c mmercially available, but in very small quantities at high cost.

As a result, there is no human parathyroid hormon available at a reasonable price t supply the many potential medical, agricultural and industrial applications.

During the past ten years, microbiological techniques employing recombinant DNA have made it possible to use microorganisms for the production of species-different peptides. The microorganism is capable of rapid and abundant growth and can be made to synthesize the foreign product in the same manner as bacterial peptides. The utility and potential of this molecular biological approach has already been proven by microbiological production of a number of human proteins that are now available for medical and other uses.

Parathyroid hormone (PTH) is one of the most important regulators of calcium metabolism in mammals and is also related to several diseases in humans, animals, e.g. milk fever, acute hypocalsemia and otherwise pathologically altered blood calcium levels. This hormone therefore will be important as a part of diagnostic kits and will also have potential as a therapeutic in human and veterinary medicine.

The first synthesis of DNA for human preproparathyroid hormone was described by Hendy, G.N., Kronenberg, H.M., Potts, Jr. J.T. and Rich, A. 78 Proc. Natl. Acad. Sci. 7365-7369 (1981). DNA complementary in sequence to PTH mRNA was synthesized and made double stranded (Hendy et al. supra). This cDNA was cloned in pBR 322 DNA and E. coli 1776 was transfected. Of the colonies with correct antibiotic resistance, 23 out of 200 clones were identified as containing specific human PTH cDNA inserts. However, none of the 23 human PTH clones contained the full length insert (Hendy et al., supra). Later Breyel, E., Morelle, G., Auf'mkolk, B., Frank, R., Blocker, H. and Mayer, H., Third European Congress on Biotechnology, 10-14 September 1984, Vol. 3, 363-369 described the presence of the human PTH gen in a fetal liver genomic DNA library c nstructed in the

phage Charon 4A. A restriction enzyme fragment of the PTH gene was recloned and transfect d into E. coli.

However, the work of Breyel, supra, demonstrated that E. coli degrades human PTH. Thus, a microorganism which shows a stable production of intact human parathyroid hormone has so far not been described. Further, parathyroid hormone has never before been isolated from yeast.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a plasmid containing DNA coding for human preproparathyroid hormone (hPTH) for insertion in Escherichia coli. It is another object of the present invention to provide a genetically engineered E. coli containing DNA coding for human preproparathyroid hormone.

A further object of the present invention is to provide a plasmid for insertion in yeast containing DNA coding for parathyroid hormone ("PTH"), It is also an object of the present invention to provide a transformed yeast containing DNA coding for parathyroid hormone including human parathyroid hormone, and from which transformed yeast, parathyroid hormone may be obtained.

Another object of the present invention is to provide new polymers having parathyroid hormone activity including PTH fragments, extension and analogs. Y t another object is to provide alternate leader sequences and secretion signal sequences which can be used in the practice of the present invention.

A still further object of the invention is to provide downstream process technology for purification of intact PTH, as well as purification of analogs, fragments and extensions.

Other objects and advantages of the present invention will become apparent as the description thereof pr ceeds.

In satisfaction of the foregoing objects and advantages, there is provided by the present invention a novel plasmid for insertion in E. c li, c ntaining DNA coding for human preproparathyroid hormone. The plasmid when inserted into E. coli functions to transform the E. coli such that the E. coli then produces multiple copies of the plasmid and thus of the CDNA coding for human preproparathyroid hormone. The plasmid for human insertion into E. Coli of the present invention and thus the transformed E. coli are distinguishable over pri r art plasmids and microorganisms, for example as described in Hendy et al., supra, in that the plasmid of the present invention contains a double start codon at the 5′ end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause a production microorganisms transformed with a plasmid containing the cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

There is further provided by the present invention a plasmid for insertion into yeast containing DNA coding for parathyroid hormone. In a preferred embodiment, this plasmid is prepared by recloning the plasmid for insertion in E. coli described above. Moreover, the invention provides a yeast transformed by said plasmid for insertion in yeast such that the yeast produces and secretes parathyroid hormone. Thus, the invention provides a method by which parathyroid hormon may be isolated from yeast culture medium. In a preferred embodiment, the transformed yeast is Saccharomyces cerevisiae. In another preferred embodiment, the parathyroid hormone is human parathyroid hormone.

By use of in vitro mutagenesis, the present invention also provides substitution of one or more amino acids in human parathyroid hormone and peptides having parathyroid hormone agonistic or antagonistic activity. Further, there are provided analogs,

fragments, or extensions of the parathyroid hormone (collectively referred to as "derivatives") which also show agonistic or antag nistic activity. Examples of these peptides have been produced as secretory products in yeast and in E. coli.

The present invention further provides different leader sequences and secretion signal sequences that may be used for the production and secretion of the PTH hormone and/or its derivatives. In at least one instance, an alternate leader sequenc provides improved production of the desired hormone or derivative.

Additionally, the invention provides a downstream process technology for purification of human parathyroid hormone and derivatives. The process involves a purification procedure yeast or E. coli medium or periplasmic solution, and consists principally of cation exchange chromatography followed by two st ps of high pressure liquid chromatography. The final product is more than 95 percent pure and can b submitted directly to N-terminal amino acid sequencing as well as amino acid composition determination.

Human parathyroid hormone (hPTH) is a key regulator of calcium homeostasis. The hormone is produced as a 115 amino-acid prepro-peptide. Before secretion the prepro part is cleaved off, yielding the 84 amino acid mature hormone. Through its action on target cells in bone and kidney tubuli, hPTH increases serum calcium and decreases serum phosphate, while opposite effects are found regarding urinary excretion of calcium and phosphate. At chronically high secret ry rates of PTH (hyperparathyroidism) bone resorption supersedes formation. However, prolonged exposure to low/moderate doses of a biologically active PTH-fragment stimulates bone formation and has also been reported to be effective in the treatment of osteoporosis by inducing an anabolic response in bone (Reeve et al. 1980 *Br Med J* 250, 1340-1344 Slovik et al. 1986 *J Bon Min*

Ros 1, 577). So far studies on intact hPTH have been hampered by the limited availability and the high price of the hormone. Hence a system for the efficient expression of hPTH in microorganisms would be very advantageous for the further progression of studies on hPTH and its role in bone biology and disease.

Poly $(A)^+$-selected RNA was isolated from human parathyroid adenomas immediately after surgery. The RNA was size-fractionated, cDNA was prepared and cloned into the PstI site of pBR322 by the GC-tailing method. Th library was screened by using synthetic oligonucleotides. Sixty-six clones of a total of 34,000 were found to be positive for both 5′ and 3′ PTH sequences. The correct identity of four of these clones was verified by DNA sequence analysis.

Employing the promoter and signal sequence of Staphyloccous aureous protein A we have expressed hPTH in *Eacherichis coli* as a secretory peptide. Immunoreactive PTH was isolated both from growth medium and periplasmic space. We obtained up to 10 mg/1 hPTH as judged by reactivity in radioimmunoassay.

hPTH was expressed in *Saccharomyces cerevisiae* after fusing hPTH cDNA to an expression vector coding for the prepro-region of the yeast mating factor α. During the secretion process, the α-factor leader sequence is cleaved off by an endopeptidase specific for a dibasic amino acid sequence and encoded by the KEX2 gene.

By hPTH-specific radioimmunoassay a significant amount of hPTH immunoreactive material was detected in the growth medium, corresponding to about 1 mg hPTH pr 1 medium, of the yeast strain FL200 transformed with fusion plasmid pαLXPTH. No immunoreactive hPTH was secreted from cells transformed with the vector pαLX.

Parallel cultures of the yeast strain FL200 transformed with one of the three expression plasmide pUCXPTH, pαUXPTH-1 and pαLXPTH with copy numbers near

unity, normal high (~30) and very high (>50) respectively were grown and both growth medium, a periplasmic fracti n and an intracellular soluble fraction were assayed for hPTH immunoreactive peptides.

The results show that the intermediate c py number gave the highest production. The produced PTH was secreted completely to the growth medium. The secreted products were concentrated from the growth medium and analyzed on SDS-PAGE. A distinct band with the same molecular weight as hPTH standard was visible on the gel.

hPTH immunoreactive material was concentrat d from the growth medium by passage through a S Sepharose Fast flow column and eluted quantitatively. Recombinant hPTH was purified by reverse phase HPLC. The column was eluted with a linear gradient f acetonitrile/trifluoroacetic acid. A major peak (fractions 32 and 33) with the same retention time as standard hPTH(1-84) was resolved into two peaks in a second HPLC urification step. The major peak from the 2.HPLC eluted exactly as standard hPTH(1-84) and co-chromatographed with hPTH(1-84) as one symmetric peak. SDS-PAGE of the peak fraction showed one band co-migrating with hPTH standard suggesting that the recombinant PTH was essentially pure. The recombinant hPTH was subjected to N-terminal amino acid analysis. We were able to determine unambiguously 45 amino acids from the N-terminal end in the E. coli protein and 19 amino acids in the yeast protein. The sequence was identical to the known sequence of hPTH. The sequence analysis indicated that the recombinant PTH was m re than 90 percent pure. The recombinant hPTH from E. coli and *Saccharomyces cerevisiae* was fully active in adenylate cyclase assay and also induced hypercalcemia in rats after injection.

We have successfully expressed biologoically active intact human parathyroid hormone as a secret ry peptide in *Escherichia coli* and *Saccharomyces*

c revisiae, and developed a down-stream purificati n technology.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows all possible variations of the DNA sequence coding for human preproparathyroid hormone.

Figure 2 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSShPTH-10.

Figure 3 shows a DNA sequence coding for human preproparathyroid hormone and having a double start codon at the 5′ terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

Figure 4 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10 with flanking sequences.

Figure 5 shows the actual amino acids sequence of the human preproparathyroid hormone for which the DNA sequence in close pSShPTH-10 codes.

Figure 6 shows the sequence of the MFα1-hPTH fusion gene with all possible combinations of the DNA coding for hPTH.

Figure 7 shows the sequence of the MFα1-hPTH fusion gene.

Figure 8. Analysis of expression products by SDS-PAGE and immonoblotting.

Saccharomyces cerevisiae transformed with a PTH cDNA carrying plasmid was grown in liquid culture medium. The secreted products were concentrated and analyzed on SDS-PAGE. Panel a shown a silver stained gel with molecular size marker (lane S), hPTH standard (lane P), and concentrated yeast growth medium (lane 1). After blotting onto a PVDF membrane, blots were probed with hPTH specific antibodies, one reactive against the aminoterminal part of the hormone (panel b), another reactive against the middle region of the hormone (panel c). Lanes in panel b and c are numbered as in panel a.

Figure 9. Purification of rec mbinant hPTH from the growth medium.

A: Chromatogram of the 1.HPLC purification

B: Chromatogram of the 2.HPLC purification of fractions 32 and 33 from panel A. The peak of the recombinant hPTH is indicated by black.

C: 2.HPLC run of 1 ug standard hPTH(1-84)

D: Co-chromatography of the recombinant PTH pack from panel B and 1 ug of standard hPTH (1-84)

E: Silver staining of SDS-PAGE of the proteins in the hPTH pack

1: recombinant hPTH, 1 ug

2: hPTH(1-84) (g), 3ug (Note HMW Impurities)

~~Figure 10. Construction of PPTH-M13-ΔEA/KQ.~~ (SEQ ID NO: 9 and 16 respectively, in order of appearance)

Figure 11. Schematic representation of the mutation introduced in the gene fusion between the yeast α-factor prepro region and the human parathyroid hormone.

Figure 12. SDS PAGE of concentrated yeast growth medium containing mutated and wild type hPTH. Aliquots of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-2^9 (lane 2) and pαUXPTH-Q26 (lane 1) were analyzed by 15% PAGE in the presence of 0.1% SDS, and visualized by silver staining as described in Experimental Protocol. Lane M shows a molecular size marker including a hPTH standard. The latter is marked with an arrow.

Figure 13. Purity of purified hPTH (1-84,Q26). Yeast growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-Q26 were concentrated and purified by reversed phase HPLC as described in Experimental Protocol. The purity of the recombinant hormone was then analyzed by analytical HPLC (Panel A) and SDS PAGE (Panel B, lane 2). In Panel B the purified hPTH (1-84,Q36) is compared with the wild type hormone purified by two runs on HPLC (lane 3). The m lecular weight market in lane M is the same as in

Figure 2. Lane 1 shows a reference PTH produced in *E. coli*.

Figure 14. Two dimensional gelelectrophoretic analysis of hPTH (1-84,Q26). An aliquot of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-Q26 was separated on an acetic acid 15% PAGE. The two main bands (band 1 and 2) migrating close to the hPTH standard were then cut out, equilibrated with SDS loading buffer and run into a second dimension 15% PAGE containing 0.1% SDS in separate lanes in triplicate. This gel was divided in three and one part was colored with silver (Panel A), one part blotted and treated with hPTH N-terminal region specific antibodies (Panel B) and one part blotted and treated with hPTH middle-region specific antibodies (Panel C). Lanes 1 and 2 show band 1 and 2, PTH_e is a reference hPTH produced in E. coli, PTH_c is a commercial hPTH reference. Lane S shows a molecular weight standard.

Figure 15. Biological activity of hPTH (1-84,Q26). Recombinant hPTH (1-84,Q26) (■) was purified on HPLC and assayed for biological activity in a hormone-sensitive osteoblast adenylate cyclase (AC) assay as described in Materials and Methods. The experiments were carried out in triplicate determinations. hPTH (1-84) from Sigma (O) and recombinant yeast hPTH (1-84) (▲) were used as references.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As indicated above, the present invention is directed to a plasmid for insertion in E. c li containing DNA coding for human preproparathyroid hormone. The invention is also directed to th resulting transformed E. coli.

The invention further is directed to a plasmid for insertion into yeast which contains DNA coding for parathyroid hormone and which is derived from the plasmid f r insertion into E. coli. Finally, the

inventi n is directed to a transformed yeast from which parathyroid hormone may be recovered.

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct siz mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated in vitro from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoreses after immuno precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase. After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating the purified first strand DNA in the presence of the Klenow fragment of E. c li DNA polymerase I. The double stranded comlementary DNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enyzmatically added to the 3 ends of the cDNA. This modified complementary DNA was annealed to restriction enconuclease PstI cleaved and d(G)-tailed vector pBR322. Resulting recombinant plasmid DNA's were transformed into E. coli KI2 BJ 5183. Positive transformants were analyzed for by colony hybridization using two different synthetic deoxyribooligonucleotides which covered th N-terminal coding region as well as the 3' non-coding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were

identical except for s me size heterogenity at the regions flanking the start codon and the XbaI site 3′ for the stop codon. One clone, pSShPTH-10, was subjected to DNA sequence analysis revealing a 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the PstI site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., supra, except for a 5 base pair deletion in front of the start codon.

Figure 2 shows the human preproparathyroid hormone DNA sequence of pSShPTH-10. This may b compared with Figure 1, which shows all possible variations of the DNA sequence for human preparathyr id hormone without the 5′ double start codon. Figure 3 shows the DNA sequence of the clone of the present invention with the flanking sequences. In a preferred embodiment, the plasmid for insertion in E. coli coding for human preproparathyroid hormone is pSShPTH-10, the DNA sequence of which, including the flanking sequence, is shown in Figure 4.

The invention further provides a plasmid for insertion into yeast containing DNA coding for parathyroid hormone. The parathyroid hormone may be human or animal parathyroid hormone, for example pig or bovine parathyroid hormone. The plasmid for insertion in yeast of the present invention may be recloned from plasmids containing DNA coding for human or animal parathyroid hormone. In a preferred embodiment, the plasmid for insertion in yeast contains DNA coding for human parathyroid hormone. As shown in the following examples, the hTPH sequence from pSShPTH-10 has been recloned and inserted in designed vectors for expression in Saccharomyces cerevisiae.

pSShPTH-10 was digested to form a 288 bp Bg1II-XbaI fragment. This fragment was then subcloned into pUC19 between the BamHI and XbaI sites. Th subclone was then digested with Dpn I, and the largest

resulting fragment was isolated. The said fragment was th n digested with SalI.

The plasmid pSSαLX5-hPTH1 that in yeast MAT cells leads to the expression and secretion of PTH was constructed in three stages:

1. Construction of the yeast shuttle vector pL4 (which replicates in both E. coli and Saccharomyces cerevisiae).
2. Cloning of a DNA fragment containing the yeast mating pheromone MFα1 gene and its insertion into the yeast shuttle vector to make the pαLX5 vector.
3. Insertion of a DNA fragment from the coding region of the hPTH gene of pSShPTH-10 into pαLX5 in reading frame with the prepro part of the MF 1 gene, thereby producing the vector pSSαLX5-hPTH1.

The shuttle vector pL4 was constructed by inserting into pJDB207, an EcoRI-AvaII fragment containing the ADHI promoter isolated from PADHO40. A SphI fragment was then deleted, resulting in a plasmid pALX1. The PstI site in the B-lactamase gene was deleted and the plasmid was partially digested withPvuI and Bg1I and ligated to a PvuI Bg1I fragment of pUC8, to form pALX2. After a further oligonucleotide insertion, the plasmid was digested with HindIII and religated to form pALX4.

Total yeast DNA from the Y288C strain was digested with EcoRI, and the 1.6-1.8 kb fragments isolated. These were ligated to EcoRI-cleaved pBR322, and E. coli was transformed. The clones were screened for MFα1 inserts by oligonucleotide hybridization. Th DNA selected thereby was then used to transform E. coli. The resulting plasmid pMFα1-1 was digested with EcoRI, made blunt ended by Klenow enzyme, and then digested with Bg1II. The MFα1 fragment was isolated, and ligated to pL5 (digested with BamHI, made blunt ended with Klenow enzyme, and digested with Bg1II) to yield pαLX5.

In order to insert the human PTH cDNA fragment into pαLX5, the pαLX5 was digested with HindIII, creating sticky ends and the site was made blunt ended with the DNA polymerase I Klenow fragment and dNTP. The pαLX5 was then digested with SalI to create a sticky ended DNA complementary to the SalI digested human PTH fragment described above.

The SalI digested human PTH fragment was then inserted into the SA1I digested pαLX5. The resulting plasmid pSSαLX5-PTH was then inserted into yeast, thereby transforming yeast so that the yeast produces and secretes intact human parathyroid hormone. In a preferred embodiment, the transformed yeast is Saccharomyces cerevisiae.

As explained above, the invention provides alternate leader sequences which may be used for the production of parathyroid hormone or derivatives thereof, as taught by the present invention. The method set forth above discloses the use of the α-factor leader sequence. However, other sequences may be used, at least one of which has been shown to process PTH with greater efficiency than does the entire α-factor leader sequence. It has been discovered that the deletion from the α-factor leader of a 12-base sequence which comprises the yeast STE13 recognition site produces a more efficient production mechanism for PTH and/or its derivatives. pSSαUXPTH-ΔEA contains the α-factor hPTH fusion gene placed between the α-factor promoter and terminator, in which the region encoding the Glu-Ala-Glu-Ala recognition sequence of the yeast STE13 aminopeptidase has been deleted. As another exampl of an alternative leader sequence, a leader sequence comprised of only the first nineteen amino acids of the α-factor is also used in the method of the present invention.

Also shown is an example of site specific mutagenesis changing the codon for the amino acid 26 in th PTH gene, thereby transforming a lysine-codon (K) to

glutamine-codon (Q) using the Muta-Gene™ in vitro mutagenesis kit from Bio-Rad. For this purpose, the plasmid pαPTH-M13-ΔEA was used to transf rm th E. coli strain CJ236. A uracil-containing single-stranded DNA which was prepared from the phage was annealed to a synthetic oligonucleotide, and second strain synthesis was carried out with T4 DNA polymerase and ligation with T4 DNA ligase. The heteroduplex DNA was transformed into the E. coli strain MV1190 to be repaired into a homoduplex by removal of uracil incorporated in the parental strand. Positive clones were verified by DNA sequencing and one of these was called pαPTH-M13-ΔEA/KQ. Finally, the entire expression cassette between a BamHI and a filled-in EcoRI site was isolated from this vector construction and inserted into the BamHI and PvuII site of the yeast shuttle vector YEp24 and this final expression plasmid was designated pSSαUXPTH-ΔEA/KQ.

A point mutation was introduced in the gene encoding the human parathyroid hormone leading t a change of the 26th amino acid from Lysine (K26) to Glutamine (Q26). When this gene was expressed and secreted in Saccharomyces cerevisiae using the α-factor fusion system, the full length hormone was found in the growth medium with no degradation products present. This contrasts the situation when the wild type gene is expressed in the same system. Then the major product is a hormone fragment hPTH(27-84), and only up to 20% of the immunoreactive secreted material is hPTH(1-84). Th yield after a two step purification of the degradation resistant hormone was 5-10 fold higher than what was obtained with the wild type hormone. The secreted hPTH(1-84,Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, the introduced mutation had no effect on the biological activity of the horm ne as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

Human parathyroid hormone (hPTH) is one of the key calcium regulating hormones in the body. The hormone is produced in the parathyroid gland as a 115 amino acid prepro-peptide that is processed during secretion to an 84 amino acid mature hormone.[1] It acts primarily on kidney and bone cells, stimulating calcium back resorption and calcium mobilization, respectively.[2-4] The hormone seems to exhibit differential catabolic as well as anabolic effects and its overall physiological action is probably to generate a positive calcium balance and enhance bone formation. The area of potential utility includes possible use in treatment of postmenopausal osteoporosis as well as in prevention of postpartum hypocalcaemia in cows. Sufficient supplies of authentic recombinant hPTH are of considerable interest to evaluate such applications.

hPTH is an easily degraded polypeptide. Already in the parathyroid gland large amounts of carboxyl-terminal PTH fragments are generated.[1] Structural studies have suggested that hPTH may contain two domains with the easily cleaved region placed in a connecting stalk between these domains.[5] N t surprisingly therefore, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms. In E. coli low expression levels combined with degraded hormone peptides of short half-life were observed.[6-8] The most successful expression system for hPTH so far is *Saccharomyces cerevisiae* where the hormone is expressed as a secretory peptide.[9] By that approach we were able to obtain significant amounts of authentic hPTH(1-84) with full biological activity. But even if conditions were found which eliminated proteolytic attacks at some sites in the putative stalk region of the hormone, a significant fraction of the secreted peptides was still cleaved after a pair of basic amino acids found in th hPTH sequence reducing the yield of full length peptide hormone. The cleavage site resembles that recognized by

th yscF protease (the KEX2 gen product).[10,11] We r asoned that the elimination of the putative yscF cleavage in hPTH could lead to a significant gain in the yield of undegraded hPTH secreted from yeast. In the present report we describe the removal of the putative yscF cleavage sites by *in vitro* mutagenesis of the hPTH coding region. When the amino acid at position 26 in hPTH was changed from Lysine (K26) to Glutamine (Q26), the major degradation product hPTH(27-84) previously observed disappeared in the growth medium and the yield of full-length hormone increased 5- to 10-fold. The secreted degradation resistant hPTH(1-84, Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, th introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

The *Saccharomyces cerevisiae* strain used for the hPTH expression was BJ1991 (*a, trp1, ura3-52, leu2, prb1-1122, pep4-3*). Yeast cells were transformed by the lithium method[12], and transformants grown at 30°C in YNBGC medium (0.67 percent yeast nitrogen base, 2 percent glucose, 1 percent casamino acids (Difco).

The paUXPTH-2 plasmid used as a reference for expression of authentic hPTH(1-84) is described.[9] In order to change the codon 26 in the hPTH gene from AAG (Lysine) to CAG (Glutamine), an a-factor hPTH gene fusion subcloned in M13 mp19 (designated M13PTH-3 in [9]) was modified by *in vitro* mutagenesis using the "Mutagene" *in vitro* mutagenesis kit" (Bio-Rad) based on the method of Kunkel et al.[13]. The mutagenizing oligonucleotide had the sequence (SEQ ID NO:9) 5'-GGCTGCGTCAGAAGCTGC-3' where all nucleotides except the ninth are complementary to the actual hPTH sequence. Positive clones were verified by DNA sequencing.[14] One of those were picked and called M13PTH-Q26. The entire expression cassette between a BamHI and a filled in

EcoRI site was finally isolated from M13PTH-Q26 and inserted between the BamHI and PvuII sit of the yeast shuttle vector YEp24.[15] This expression plasmid was designated paUXPTH-Q26. The translation product from the hPTH gene between amino acid 25 and 27 should now change from Arg-Lys-Lys to Arg-Gln-Lys.

Radioimmunoassay of hPTH in yeast culture media was carried out as described.[9],[16]. For electrophoretic analysis, yeast culture media were concentrated as previously described[9], and separated on a 15 percent polyacrylamide gel in the presence of SDS[17], and either stained with silver[18] or further analyzed by protein blotting using Immobilon PVDF Transfer Membranes (Millipore) and the buffers of Towbin et al.[19] Reference hPTH(1-84) was purchased fr m Peninsula Laboratories (USA). Protein blots were visualized as described.[9]

The concentrated medium from the Sepharose S column was subjected to further purification by reversed phase HPLC using a Vydac protein peptide C18 column (The Separation Group, Hesperia, CA, USA). The column was eluted with a linear gradient of acetonitrile/0.1 percent trifluoroacetic acid.

Proteins to be sequenced was purified either by HPLC as described above or by SDS polyacrylamide gelelectrophoresis followed by blotting onto polyvinylidene difluoride membranes.[20] Automated Edman degradation was performed on a 477A Protein Sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA, USA). All reagents were obtained from Applied Biosystems.

The adenylate cyclase stimulating activity of the recombinant hPTH was assayed as previously described[9,21,22] hPTH(1-84) from Sigma was used as reference.

Different strategies could be envisaged to avoid the degradation of parathyroid h rmon during expression in heterologous organisms. One recently

reported strategy is to express intracellularly in E. coli a cro-lacZ-hPTH fusion protein that subsequently is cleaved by strong acid to give proline-substituted hPTH.[23] However, since secretion of the hormone in yeast seems to be a more efficient way of producing a correctly processed hormone, and also is superior with respect to downstream processing, we rather adopted a strategy to improve this system. Only one major cleavage site is used during secretion in yeast when the cells are grown under proper conditions: after a pair of basic amino acids in position 25 and 26 in the hPTH sequence. This cleavage site resembles that recognized by the yscF protease (the KEX2 gene product). We reasoned that a substitution of a glutamine for the lysine 26, as illustrated in Fig. 11, ought to b a structurally conservative change that should exclude the hormone as a substrate for the yscF protease.

The yeast strain BJ1991 was transformed with the plasmids paUXPTH-Q26 containing the mutated hPTH coding region. One transformant was grown in YNBGC medium lacking uracil and the cell free medium was concentrated and analyzed in different gel systems. Figure 12 shows a silver-stained SDS polyacrylamide gel where concentrated medium from paUXPTH-Q26 transformed cells (mutated hPTH, lane 1) is compared with that from paUXPTH-2 transformed cells (wild type hPTH, lane 2). In the latter case the strongest band has a molecular mass lower than the standard hPTH, and previous microsequencing has shown that it corresponds to th hormone fragment hPTH(27-84). In the lane with the mutated product (lane 1), this band is absent showing that the cleavage between amino acid 26 and 27 has been totally eliminated as a result of the mutation. Now the major product is a polypeptide that migrates clos to the full length hPTH standard. Consistently, this band had a migration slightly faster than the standard in an anionic gel system and a migration slightly slower than the standard in a cationic gel system in accordance with

the single charge difference between the mutated (one positive charg less) and the wild type hormone. In addition to the main product a few weaker bands were present of apparently higher m lecular mass which might be O-glycosylated forms of the hormone.

This hPTH(1-84,Q26) candidate was further analyzed by two dimensional gel electrophoresis and protein blotting. In the first dimension acetic acid/urea gel a simple pattern with mainly two bands was found. These were cut out and run on a second dimension SDS polyacrylamide gel. The silver stained second dimension gel as well as two protein blots probed with different PTH antibodies, are shown in Figure 14. The hPTH(1-84,Q26) candidate migrating closest to the hPTH standard in both dimensions, reacted with two hPTH specific antibodies raised against N-terminal region and the middle/C-terminal region of the hPTH respectively.

The nature of the hPTH(1-84,Q26) candidate was finally confirmed by N-terminal amino acid sequencing, both directly on the polypeptide band after blotting onto a PVDF membrane filter, and after purification on reversed phase HPLC. Correct amino-terminal sequenc was found in both cases. Furthermore, the expected change from lysine to glutamine in position 26 was confirmed by sequencing through this position.

Since the elimination of the internal cleavage of the secreted hPTH leads to fewer polypeptides with similar properties in the growth medium, this form of the hormone could also be isolated by a simplified purification procedure. Already in the first concentration step using a Sepharose S column, a certain purification is achieved. All hPTH immunoreactive material is retained, but some high molecular weight material is removed in the pH6 wash of the Sepharose S column. This first concentrated eluate already contained more than 80 percent hPTH(1-84, Q26). Then, a single run on a reversed phase HPLC C18 column, was enough to give near homogeneous hPTH(1-84, Q26). The

purity was checked both by SDS polyacrylamide gel lectrophoresis and sensitive silver-staining, and by analytical HPLC as illustrated in Figure 13A. A single peak is found in the chromatogram (Figure 13A), and a single band with only a trace of a closely migrating hPTH band (probably an O-glycosylated form of the hormone) could be seen in the SDS polyacrylamide gel (Figure 13B). When the yield of pure full length mutated hormone was compared with that of the wild typ , 5 to 10 fold higher yields were usually achieved. This is consistent with our previous estimate of the fraction of full length hormone (up to 20 percent) obtained when the wild type is expressed.[9/]

The biological activity of the secreted hPTH(1-84, Q26) was tested in a hormone-sensitive osteoblast adenylate cyclase assay.[9,21,22/] The purified hPTH(1-84, Q26) was analyzed for its ability to stimulate the adenylate cyclase activity of OMR 106 osteosarcoma cells above the basal level. The quantitative analysis shown in Figure 15, clearly demonstrates that hPTH(1-84, Q26) has a stimulatory effect comparable to that of a commercial hPTH control. The stimulation curve practically coincides with that of purified recombinant wild type hPTH(1-84). Consequently, no difference in biological activity could be detected between the wild type hormone and the degradation resistant mutated hormone.

We have shown that the easily degraded human parathyroid hormone can be expressed in a correctly processed and intact form in Saccharomyces cerevisiae after the introduction of a single, structurally conservative mutation in the 26th amino acid of th hormone. The increase in final yield of pure full length hormone is 5- to 10-fold compared to what is obtained with wild type hormone expressed in the same system. The mutation also simplifies the downstream purification of the hormone. A c ncentration step

followed by a single HPLC run was enough to give near homogeneous recombinant hormone.

We have previously described conditions of growth that eliminates secondary cleavages in the protease sensitive "stalk" region of the hormone [9]. Here we describe how the final dibasic cleavage site can be eliminated. After introduction of the mutation, a form of the hormone is produced that totally resists the frequent cleavage found in the wild type hormone aft r the Arg25-Lys26 motif. The possible internal cleavag at putative dibasic amino acids is one of the severe drawbacks of the α-factor secretion system. To our knowledge this is the first reported case where this problem has been successfully overcome.

Previous reports have shown that th biological activity of the hormone resides in the first third of the molecule in a minimum structure comprised of amino acids 1-27. Furthermore, the triple basic amino acid motif from position 25-27 seems to be conserved between the bovine[25], porcine[26] and human hormone[27]. It was therefore not obvious that the introduction of a mutation in position 26 would not destroy the biological activity of hPTH. However, no difference between the recombinant hPTH products could be detected in the adenylate cyclase assay, showing that the introduced mutation does not affect the biological activity of the hormone.

hPTH is a multifunctional hormone with many potential uses, for example in diagnostics and as a drug in veterinary medicine. A fragment of hPTH together with $1,25(OH)_2$ vitamin D_3 has also been reported t induce bone formation in humans [27, 28], and one of the major areas of potential use of a recombinant hPTH is therefore in the treatment of osteoporosis. To evaluate such applications, sufficient supplies of recombinant hPTH are essential. In the present report we have described what we believe is the most efficient way of

producing full length biologically active parathyr id hormone so far.

Moreover, the method of the present inventi n may be used to produce parathyroid hormone derivatives having parathyroid hormone agonistic or antagonistic activity. These derivatives include hormone anal gs, such as the example described above in which the lysine at position 26 is substituted with glutamine, or may be fragments or extensions of the hormone, i.e., polypeptides having parathyroid hormone agonist or antagonist activity which are respectively shorter or longer than the hormone itself. Parathyroid hormon agonistic effect in this connection will be demonstrated by activation of adenylyl cyclase in bone cells and kidney cells. The <u>in vivo</u> effects of such activity mimic the effects of native parathyroid hormone with respect to plasma calcium concentration alterations as well as the well known hormonal actions on calcium and phosphate re-absorption and excretion in the kidney. Furthermore, the PTH derivatives of the present invention having agonist activity shall also have the capacity to reduce the alkaline phosphatase activity of certain osteoblast cell lines, and stimulate ornithine decarboxylase activity bone cells (UMR 106 cells) or chicken condrocytes and stimulate DNA synthesis in chicken condrocytes. Moreover, the derivatives shall have the capability of blocking the action f parathyroid hormone itself or of any of the other agonist derivatives.

The invention also provides alternate secretion signal sequences for the secretion of the PTH hormone or its derivatives from yeast. As disclosed above, parts of the MFα1 gene may be inserted into the plasmid of the present invention to cause the yeast to secrete the intact PTH hormone or derivatives. However, other signal sequences will also function in the methods of the present invention. The process of protein secretion requires the protein to bear an amino-terminal

signal peptide for correct intracellular trafficking, the sequence of which is t rmed "signal sequence". Two classes of signal sequences will function in the plasmids of the present invention, and will cause secretion of the PTH hormone or derivative from yeast: "optimalized consensus signal sequences" and other functional signal sequences. An "optimalized consensus signal sequence" is any amino-terminal amino acid sequence that is composed of the following three parts:

1. An amino-terminal positively charged region. The size of this region may vary from 1-20 amino acids. The only specific characteristic is a positive charge at physiological pH conferred by the presence of one to three basic amino acids (Lys or Arg).

2. A hydrophobic core region. The size of this region may vary from 7-20 amino acids, and it is predominantly composed of hydrophobic amino acids (Phe, Ile, Leu, Met, Val, Tyr, or Trp).

3. A polar COOH-terminal region composed of five amino acids (from position -5 to -1 relative to the cleavage site) that defines the cleavage site. The specific character of this region is that the amino acid in position -1 must be a small neutral amino acid (Ala, Ser, Gly, Cys, Thr, r Pro), and that the amino acid in position -3 must be either a hydrophobic amino acid (Phe, Ile, Leu, Met, Val) or a small neutral amino acid (Ala, Ser, Gly, Cys, Thr, or Pro).

See von Heijne, G. (1983) "Patterns of Amino Acids near Signal-Sequence Cleavage Sites." Eur. J. Biochem. 133, 17-21, and von Heijne, G. (1985) "Signal sequences. The limits of variation." J. Mol. Biol. 184, 99-105. However, Kaiser, C.A., Preuss, D., Grisafi, P., and Botstein, D. (1987) "Many Random Sequences Functionally R place the Secretion Signal Sequence of Yeast Invertase." Science 235, 312-217, found the specificity with which signal sequences were recognized in yeast to

be low and that any amino-terminal peptide with a hydrophobicity abov some threshold value would function. Therefore, "functional signal sequence" is defined as any amino-terminal amino acid sequence that can direct secretion in yeast even if it does not fit all the criteria of an optimal signal sequence.

Specific examples of signal sequences functional in yeast that conform to the description of an optimal signal sequence are:

1. Met,Lys,Ala,Lys-Leu,Leu,Val,Leu,Leu,Thr,Ala, Phe-Val,Ala,Thr,Asp,Ala (Jabbar, M.A., and Nayak, D.P. (1987) "Signal Processing, Glycosylation, and Secretion of Mutant Hemagglutinins of a Human Influenza Virus by Saccharomyces cerevisiae." Molec. Cell. Biol. 7, 1476-1485.) from a human influenza virus hemagglutinin.

2. Met,Arg,Ser-Leu,Leu,Ile,Leu,Val,Leu,Cys,Phe, Leu,Pro-Leu,Ala,Ala,Leu,Gly (Jigami, Y., Muraki, M., Harada, N., and Tanaka, H. (1986) "Expression of synthetic human-lysozyme gene in Saccharomyces cerevisiae: use of a synthetic chicken-lysozyme signal sequence for secretion and processing." Gene 43, 273-279.) from chicken lysozyme.

3. Met,Arg,Phe,Pro,Ser-Ile,Phe,Thr,Ala,Val,Leu, Phe,Ala,Ala-Ser,Ser,Ala,Leu,Ala (Ernst, J.F. (1988) "Efficient Secretion and Processing of Heterologous Proteins in Saccharomyces cerevisiae is mediated solely by the Pre-Segment of α-factor Precursor." DNA 7, 355-360. Kurjan, J. and Herskowitz, I. (1982) "Structure of a Y ast Pheromone Gene (MFa): "A putative α-factor Precursor contains four Tandem Copies of Mature α-factor". Cell 30, 933-934.) from yeast α-factor precursor.

A specific example of signal s quences functional in yeast that conforms to the description of

a functional signal sequence is Met,Asn,Ile,Phe,Tyr,Ile,Phe,Leu,Phe,Leu,Ser,Phe,Val-Gln, Gly, Thr,Arg,Gly. Baldari, C., Marray, J.A.H., Ghiara, P., Cesareni, G., and Caleotti, C.L. (1987) "A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1B in Saccharomyces cerevisiae." EMBO J. 6. 229-234. from Klyveromyces lacis killer toxin.

Finally, the invention provides three different steps which taken together, represent an effective and convenient procedure for purification of human recombinant parathyroid hormone (PTH). A cation exchange chromatography using S-Sepharose column as described in the text, washed at pH 6 and eluted at pH 8.5. The immunoreactivity of the intact PTH migrates within the peak.

Figure 9 shows high performance liquid chromatography (HPLC) of hPTH which was eluted with trifluoraecetic acid and a linear gradient of acetonitril of 35-60%. The position of intact hPTH is indicated in the second HPLC step the acetonitril gradient has been changed to 40-45% and intact hPTH elutes as one symmetrical peak.

Although the methods of making the invention disclosed herein are shown in detail, these methods are presented to illustrate the invention, and the inventi n is not limited thereto. The methods may be applied to a variety of other plasmids containing DNA coding for human or animal PTH to produce the plasmids f r insertion in yeast of the present invention.

The plasmids of the present invention and transformed microorganisms were produced as set forth in the following examples.

EXAMPLE 1

Isolation of mRNA and synthesis of complementary DNA (cDNA) of human parathyr id hormone.

Starting material for the invention was parathyroid adenomas obtained fr m patients by surgery.

The parathyroid tissue was placed on dry ice directly after removal and transp rted to a laboratory for preparation f RNA. The frozen tissue was hom genized with an ultra Turax homogenizer in the presence of 4 M Guanidinium thiocyanate and the RNA content was recovered by serial ethanol precipitations as described by Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., 18 Biochemistry 5294-5299 (1979). The RNA preparation was applied to oligo d(T) cellulose affinity chromatography column in order to enrich for poly(A) containing mRNA. The poly(A) rich RNA was further enriched for parathyroid hormone (PTH) mRNA sized RNA by ultracentrifugation through a 15-30% linear sucrose gradient. The resulting gradient was divided into 25 fractions and every third fraction was assayed for PTH mRNA content by in vitro translation followed by immunoprecipitation with anti PTH antiserum (Gautvik, K.M., Gautvik, V.T. and Halvorsen, J.F., Scand. J. Clin. Lab. Invest. 43, 553-564 (1983)) and SDS-polyacrylamide gel electrophoresis (Laemmeli, U.K., 227 Nature 680 (1970)). The RNA from the fractions containing translatable PTH mRNA was recovered by ethanol precipitation. This RNA, enriched for PTH mRNA, was used as a template for cDNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase for catalysis of the reaction (Maniatis, T., Fritsch, E.F. and Sambrook, J., Molecular Cloning pp. 230-243 (1982)). After first strand synthesis, th RNA templates were removed by alkali hydrolysis. The second strand cDNA was synthesized by incubating the purified first strand cDNA in the presence of the Klenow fragment of E. coli DNA polymerase I (Maniatis, supra). This in vitro synthesized double stranded cDNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 (Maniatis, supra). The blunt ended double stranded cDNA was size fractionated over a 15-30% neutral sucrose gradient. The size distribution of each fraction was estimated by agarose

gel electrophoresis together with known DNA fragment markers. Fractions containing cDNA larger than approximately 500 base pairs were pooled and the cDNA content was collected by ethanol precipitation.

EXAMPLE 2

Cloning of cDNA PTH in plasmid pBR 322 and transformation of E. coli K12 BJ5183.

An approximate 20 base long d(C)-tail protrusion was enzymatically added to the 3' ends of the cDNA by the action of terminal deoxynucleotidyl transferase (Maniatis, supra). The d(C)-tailed cDNA was annealed to restriction endonuclease Pst I cleaved and d(G)-tailed vector pBR322 and the resulting recombinant plasmid DNA's were transformed into E. coli K12 BJ 5183 cells which were made competent by the method of Hanahan, D., 166 J. Mol. Biol. 166, 557-580 (1983). A total of 33,000 transformants were analyzed for PTH cDNA content by colony hybridization (Hanahan, D. and Meselson, Gene 10, 63 (1980)).

Two to three thousand transformants were plated directly on each 82 mm diameter nitrocellulose filter, placed on top of rich medium agar plates containing tetracycline, and incubated at 37 degr es Centigrade until approximately 0.1 mm diameter colonies appeared. Duplicate replicas of each filter was obtained by serial pressing of two new filters against the original filter. The replica filters were placed on top of new tetracycline containing agar plates and incubated at 37 degrees Centigrade until approximately 0.5 mm diameter colonies appeared. The master filter with bacterial colonies was kept at 4 degrees Centigrade placed on top of the agar plate and the duplicate replica filters were removed from the agar plates and submitted to the following colony hybridization procedure.

EXAMPLE 3

Characterization of bacterial clones containing recombinant cDNA PTH and of the DNA sequence of clone pSSHPTH-10.

The cells in the respective colonies were disrupted in situ with alkali and sodium chloride leaving the DNA content of each bacterial clone exposed. The procedure allows the DNA to bind to the filter after which it was neutralized with Tris-buffer and dried at 80 degrees Centigrade. The majority of cell debris was removed by a 65 degree Centigrade wash with the detergent sodium dodecylsulphate (SDS) and sodium chloride leaving the DNA bound to the filters at the position of the former bacterial colonies. The filters were presoaked in 6xSSC (0.9 M NaCl, 0.09M Na-citrate), 1x Denhart's solution (0.1 g/ml FIcoll, 0.1 g/ml polyvinyl pyrrolidone, 0.1 g/ml bovine serum albumin), 100 g/ml herring sperm DNA, 0.5% SDS and 0.05% sodium pyrophosphate for 2 hours at 37 degrees Centigrade (Woods, D.E. 6 Focus Vol. No. 3. (1984)).

The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 g/ml tRNA and 0.05% sodium pyrophophate) supplemented with 32P-labelled DNA probe. (Woods supra).

The DNA used as a hybridization probe was one of two different synthetic deoxyribo oligonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, supra. The first probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading TACTATGGACGTTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the restriction endonuclease XbaI located 31 nucleotides downstream of the termination c don and consisted of the nucleotide sequence, CTCAAGACGAGATCTGTCACATCC. (SEQ ID NO:11)

Labelling was carried out by transfer of 32 P from 32 P-γ-ATP to the 5′ end of the oligonucleotides by th action of polynucleotide kinase (Maxam, A.M. and Gilbert, W., 65 Methods Enzymol., 499 (1980)).

The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica filters. All those were picked from the original filters with the stored cDNA library and amplified for indefinitive storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a m re detailed analysis by restriction endonuclease mapping, showing that all were identical except for some siz heterogenity at the regions flanking the start codon and Xba I site, respectively.

EXAMPLE 4

Clone pSShPTH-10.

One clone, pSShPTH-10, was subjected to DNA sequence analysis according to the method of Maxam and Gilbert, supra. This clone consists of a 432 base pair long PTH cDNA sequence inserted in the Pst I site f pBR322 having 27 G/C base pairs at the 5′ end and 17 G/C base pairs at the 3′ end. The complete DNA sequence of the cDNA insert of pSSHPTH-10 is shown in Figure 4. It is identical to the sequence of Hendy, et al., supra except for a five base pair deletion right in front of the start codon, changing the published (Hendy, supra) start-stop (ATGTGAAG) signal (deletion is underlined) preceding the used start codon (ATG) to a double start signal (ATGATG).

EXAMPLE 5

Construction of the yeast shuttle vector pL4.

Before the hPTH-yeast-expression project was initiated, a family of general yeast expression vect rs were developed. One of thes , pL4, later was used t make pSS LX5-hPTH1, as described below:

The plasmid pJDB207, construct d by Beggs, J.D., "Multipl -c py yeast plasmid vectors," Von Wettstein, D., Friis, J., Kielland-Brandt, M. and Stenderup, A. (Eds) Molecular Genetics in Yeast (1981), Alfred Benzon Symposium Vol. 16, 383-390, was chosen as the basis for the general expression vectors. It contains an EcoRI fragment of the yeast 2 micron DNA inserted into the pBR322 derivative pAT153. It also contains the yeast LEU2 gene. The copy number of pJDB207 in yeast cir$^+$ cells is very high relative t that of other plasmids and it is unusually stable after non-selective growth in a cir$^+$ strain, Parent, S.A., Fenimore, C.M., and Bostian, K.A. "Vector Systems for the Expression, Analysis and Cloning of DNA Sequences in S. cerevisiae". 1 Yeast 83-138 (1985); Erhart, E. and Hollenberg, C.P., "The Presence of a Defective LEV2 Gene on 2 Micron DNA Recombinant Plasmids of Saccharomyces cerevisiae is Responsible for Curing and High Copy Number," 156 J. Bacteriol. 625-635 (1983). These properties are related to a partial defective promoter in the selective marker gene LEU2 (often named LEU2d, d for defective), Erhart et al., supra, which is not changed in the following constructs.

A 1260 base pair EcoRI-AvaII fragment containing the ADHI promoter was isolated from th plasmid pADH040. After a fill in reaction with th Klenow fragment of DNA polymerase I and all four dNTPs, BamHI linkers were attached and the fragment was cloned into the unique BamHI site of pJDB207. From the plasmid with the promoter in a counterclockwise direction, a 1050 base pair SphI fragment was then deleted (from the SphI site in pJDB207 to the SphI site in the promoter fragment) leaving only a single BamHI site. This plasmid was designated pALX1.

Then the PstI site in the B-lactamase gene of pALX1 was eliminated without inactivating the g ne. pALX1 was digested to completion with PstI and nuclease S1 to destroy the PstI site, and then subjected to a

partial digestion with PvuI Bg1I. At the same time a 250 base pair PVUI Bg1I fragment was isolated from pUC8, Vierira, J. and Messing, J. 19 Gene 259 (1982), that contains the corresponding part of a B-lactamase without a PstI site. This was ligated to the partially digested pALX1. In all the ampicillin resistant clones isolated the B-lactamase gene had been restored by incorporating the pUC8 fragment. This plasmid was called pALX2.

The following oligonucleotide was purchased from Prof. K. Kleppe, University of Bergen, and insert d into the BamHI site of pALX2:

```
     Bg1II   *     * *  HindIII                    (SEQ ID NO: 12)
GATCAGATCTGCAGGATGGATCCAAAGCTT      : initiation codon
  TCTAGACGTCCTACCTAGGTTTCGAACTAG *  : optimal ATG context (SEQ ID NO: 13)
       PstI     BamHI
```

Plasmids with the proper orientation wer isolated and designated pALX3.

Finally the pALX3 was digested with HindIII and religated to delete a HindIII fragment of 480 base pairs. The resulting vector is called pALX4.

pL4 is a derivative of pALX4 in which the ADHI promoter is deleted. pL4 was used as a basis for the insertion of other promoters. pALX4 was first digested with Bg1II and Sa1I. The resulting sticky ends were filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs follqwed by religation. By this treatment the ADHI promoter is eliminated and the Bg1II site regenerated to give the vector pL4.

EXAMPLE 6

Construction of pαLX5.

The gene for the yeast mating pheromone MFα1 was first cloned by Kurjan, J. and Herskowitz, I., "Structure of a Yeast Pheromone Gene (MFα): A Putative -factor Precursor Contains Four Tandem Copies of Mature -factor". 30 Cell, 933-943 (1982). The published sequence was used to reclone the MFα1 gene. Total yeast DNA from the strain Y288C was digested with EcoRI and digestion products in the size range from 1.6 to 1.8 kb were isolated from a preparative agarose gel. These

were then ligated to dephophorylated EcoRI cleaved pBR322 and used to transform E. coli BJ5183. The resulting clones were screened for MFα1 gene inserts by hybridization to a labeled oligonucleotide of the following composition: (SEQ ID NO:14)

TGGCATTGGCTGCAACTAAAGC

DNA from purified positive clones was then used to transform E. coli JA221 from which plasmid DNA was prepared. The plasmid used in the following constructs was pMFα1-1.

pMFα1-1 was digested with EcoRI, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglII. The 1.7 kb MF 1 gene fragment was isolated from an agarose gel. Before inserting it into the yeast shuttle vector, the HindIII site of pL4 was eliminated by HindIII digestion, Klenow fill-in reaction and religation to give the pL5 shuttle vector. pL5 was digested with BamHI, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglII. After purification on gel it was ligated to the MFα1 fragment to give the expression vector pαLX5.

EXAMPLE 7

Construction of pSS LX5-HPTH1.

A 288 base pair BglII XbaI fragment from the pSSHPTH-10 plasmid was isolated and subcloned in pUC19 using the BamHI and XbaI site of this vector. This subclone designated pUC-HPTH, was digested with DpnI and the largest fragment isolated. This fragment was then digested with SalI and the smallest of the two resulting fragments was again isolated, yielding a sticky end on the SalI cut side and a blunt end at the DpnI cut side.

pαLX5 was digested with HindIII, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with SalI. After purification from gel, it was ligated to the hPTH fragment described above. The resulting clones had the HindIII site regenerated verifying that the reading

frame was correct. This plasmid called pSSαLX5-hPTH1. The sequence of the MFα1-hPTH fusion gene is shown in Figur 6.

EXAMPLE 8

Expression And Secretion Of HPTH In Yeast.

The yeast strain FL200 (, ura3, leu2) was transformed with the plasmids pαLX5 and pSSαLX5-hPTH1 using the spheroplast method. One transformant of each kind was grown up in leu⁻ medium and aliquots of th cell-free medium were analyzed by SDS-PAGE developed by silver-staining. Two major bands were seen in th medium from the pSSαLX5-H1 transformant that were not present in the medium from the p LX5 transformant: one band of approximately 9000 daltons, the expected size of HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MFα1-hPTH fusion product. Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

The examples are included by way of illustration, but the invention is not limited thereto. While the above examples are directed to providing a S. cerevisiae which produces and excretes human parathyroid hormone, the method of the present invention may be applied to produce a plasmid containing DNA coding for parathyroid hormone from any species. Further, said plasmid may be inserted into any species of yeast. The invention thus is not limited to S. cerevisiae.

The cloned human parathyroid hormone produced by the yeast of the present invention has a variety f known and potential uses. For example, it is current medical theory that human parathyroid hormone will b highly effective in treating osteoporosis. Genetically engineered parathyroid hormone may be useful in an analytical kit for measuring parathyroid hormone levels in humans and animals. Human parathyroid hormone or fragments thereof may also be used for treatment of humans or animals displaying reduced or pathologically

altered blood calcium levels. It is anticipated that many other uses will be discovered when genetically engineered parathyroid hormone is available in large quantities, for example as a result of the present invention.

EXAMPLE 9

Deletion of the STE 13 recognition sequence positioned N-terminal for the parathyroid hormone.

In order to delete the STE13 recognition sequence (SEQ ID NO:15) (Glu-Ala-Glu-Ala) located immediately N-terminal to PTH by site directed in vitro mutagenesis of the fusion gene, a 1495 bp XbaI fragment was isolated from pSSαLX5-PTH. This contained the α-factor promot r (MFαprom), the α-factor leader sequence (PP) and the human PTH gene (hPTH) including the stop codon. The fragment was subcloned into M13 mp19 to give the plasmid p PTHx-M13. An oligonucleotide with the sequence GGATAAAAGATCTGTGAG (SEQ ID NO:16) was made where the first t n nucleotides are complementary to the sequence of the α-factor leader in pαPTHx-M13 just proceeding th Glu-Ala-Glu-Ala coding region, and the last eight nucleotides are complementary to the beginning of the human PTH sequence. When this oligonucleotide was annealed to single-stranded DNA prepared from the recombinant phage, the following heteroduplex was generated:

oligonucleotide (SEQ ID NO:16): 5'-GGATAAAAGATCTGTGAG-3'

p PTHx-M13 (SEQ ID NO:17): 3'-CCTATTTTCTAGACACTC-5'

C A

T G [to be removed]

CCGACTTC

translation product (SEQ ID NO:18): ..AspLysArgSerVal.. (upper)

..AspLysArgGluAlaGluAlaSerVal... (SEQ ID NO:19) (lower)

After second strand synthesis and ligation with the Klenow fragment of DNA polymerase I and T4 DNA ligase, closed circular heteroduplex DNA was isolated by s dimentation through an alkaline sucrose gradient as described in Carter, P., Bedouelle, H., Waye, M.M.Y., and Winter, G. (1985) "oligonucleotide site-directed

mutagenesis in M13. An experimental manual," MRC Laboratory of Molecular Biology, Cambridge CB2 2QH., the disclosure of which is hereby incorp rated by reference. The heteroduplex DNA was used to transform a BMH 71-18 mutL strain of E. coli defective in mismatch repair (kindly provided by Dr. G. Winter). Positive clones with the looped out sequence, 3' CTCCGACTTCGA-5' (Seq ID NO:25) deleted were identified by colony hybridization using the mutagenizing oligonucleotide as the probe and by DNA sequencing. The plasmid in these clones was designated pαPTHx-M13ΔEA.

The α-factor transcription terminator was then inserted into one of the positive M13 clones as a SalI HindIII fragment isolated from pMFα1, to give a plasmid called pαPTH-M13-ΔEA. The entire expression cassette between a BamHI and a filled-in EcoRI site was finally isolated from pαPTH-M13-ΔEA and inserted between the BamHI and PvuII site of the yeast shuttle vector YEp24 by the method described in Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchc mb, D.T., Struhl, K., and Davis, R.W. (1979) Gene 8, 17-24, which is hereby incorporated by reference. This expression plasmid was designated pSSαUXPTH-ΔEA.

EXAMPLE 10

Conversion of intact hPTH by substitution of lysine with glutamine at position 26, designated PTH_{Q26}

In order to change the amino acid at position 26 in the human PTH from lysine to glutamine, the fusion gene in pαPTH-M13-ΔEA was further modified by *in vitro* mutagenesis using the "Muta-gene™ *in vitro* mutagenesis kit" obtained from Bio-Rad based on the method of Kunkel; Kunkel, T.A., Roberts, J.D., and Sakour, R.A. (1987) "Rapid and efficient site-specific mutagenesis without phenotypic selection" in Methods of Enzymologi, (Wu, R., and Grossman, L., eds.) vol. 154, pp 367-381, which is hereby inc rporated by reference. The E. coli strain or CJ236 (dut, ung, thi, rel A; pCJ105 (Cm^r)) was

transformed with the pαPTH-M13-ΔEA plasmid. The single-stranded DNA that was prepared from the phage contained a number of uracils in thymin positions as a result of the dut mutation (inactivates dUTPase) and the ung mutation (inactivates the repair enzyme uracil N-glycosylase). An oligonucleotide with the sequence GGCTGCGTCAGAAGCTGC was made where all nucleotides except the ninth are complementary to an internal PTH sequence in pαPTHx-M13. When this oligonucleotide was annealed to the single-stranded DNA, the following heteroduplex was generated:

```
                                        C
                                       / \
oligonucleotide:          5'-GGCTGCGT CAGAAGCTGC-3'
pαPTH-M13-ΔEA      3'....CCGACGCA TCTTCGACG....5'
                                       \ /
                                        T
Translation product  ...LeuArgGlnLysLeu...  (upper)
                     ...LeuArgLysLysLeu...  (lower)
```

After second strand synthesis and ligation with T4 DNA polymerase and T4 DNA ligase, the heteroduplex DNA was transformed into the E. coli strain MV1190 ((lac-pro AB), thi, sup E, Δ(srl-rec A)306::Tn10(tet^r)[F': tra D36, pro AB, lac I^q Z M15]) which contains a proficient uracil N-glycosylase. During the repair process in this host eliminating the uracils in the paternal strand, the in vitro synthesized strand will serve as a repair template conserving the mutation. Positive clones were verified by DNA sequencing. One of those were picked and called pαPTH-M13-ΔEA/KQ. The entire expression cassette between a BamHI and a filled-in EcoRI site was finally isolated from pαPTH-M13-ΔEA/KQ and inserted between the BamHI and PvuII site of the yeast shuttle vector YEp24. This expression plasmid was designated pSSαUXPTH-ΔEA/KQ.

EXAMPLE 11

Expression and secreti n f hPTH$_{Q26}$ in yeast.

The yeast strain BJ1991 (α,Leu2,wa3-52,trpl,pr67-112,pep4-3) was transformed with the

plasmids pSSαUXPTH-ΔEA and pSSαUXPTH-ΔEA/KQ using the lithium method. One transformant of each kind was grown in medium lacking uracil and the cell free medium was analyzed as described below.

EXAMPLE 12

Purification of heterologous hPTH from yeast medium concentration and purification by S-Sepharose R fast flow.

Samples of cell free yeast medium (1-10 l) (containing 1% Glucose, 2% casamino acid, 134% yeast nitrogen base w/o amino acids, 60 mg/ml trp, 180 kg/l) were adjusted to pH 3.0 and run through a 10mlx10 S-Sepharose^{R} (Pharmacia AB) fast flow column, pre-equilibrated with 0.1M glycine pH 3.0. The loaded column was eluted by 13 ml 0.1M acetic acid buffered to pH 6.0, followed by 20 ml 0.1M $NH_4HC)_3$ pH 8.5. The peptides eluted from the column were monitored by a Pharmacia optical unit (Single path monitor UVI, Pharmacia AB, Uppsala, Sweden) at 280nm, and collected in 2ml fractions by an LKB 2070 Ultrorac II fraction collector (LKB, AB, Bromma, Sweden).

EXAMPLE 13

Purification by HPLC.

Collected fractions from S-Sepharose fast flow chromatography were subjected to further purification by reversed phase HPLC using a 25 cm x 4.2 cm Vydac protein peptide C18 column (The Separations Group, Hesperia, California, USA) and an LDC gradient mixer, LDC contamertric pumps model I and III with a high pressure mixing chamber and LDC spectromonitor III with variable UV monitor. (LDC Riviera Beach FL, USA). Chromatograms were recorded by a Vitatron 2 channel recorder. The analytical conditions were as follows:

First HPLC purification step:

Gradient: 35-60%B, 60 min., linear

A: 0.1% trifluoroacetic acid (TFA)

B: 70% acetonitril in A (ACN)

Flow: 1.0 ml/min

Detection: UV 220 nm

Sec nd HPLC purification step:

Same as first step, with the following modification:

Gradient: 40-45%B 60 min; linear.

EXAMPLE 14

Assessment of the $hPTH_{Q26}$ product.

This PTH analog was verified to represent th designed product by N-terminal amino acid sequence analysis including amino acid no. 30 and shown to be hPTH identical except for the lysine to glutamine substitution at position 26.

Moreover, the resulting amino acid composition had the expected alterations, in that the sequence contained one residue less of lysine and one residue more of glutamine.

Its biological activity was assessed after purification by testing the effect of synthetically bought human parathyroid hormone fitures in comparison to the recombinant analogue which was equally potent in stimulating the adenylyl cyclase of bone cell membranes from rat calveria as well as from an osteosarcoma cell line.

EXAMPLE 15

Additional examples of amino acid substitutions by site specific in vitro mutagenesis.

By the above method, it is possible to obtain any amino acid substitution or sequences of amino acid alterations in the PTH molecule. By use of the "Muta-Gene" in vitro mutagenesis kit" and synthetic oligonucleotides with the desired sequence corresponding to the amino acid alteration(s), this may be carried out. Each of these oligonucleotides can be annealed to the single-stranded DNA in order to generate a hetroduplex as indicated above.

Followed by second strand synthesis and ligation with T4 DNA polymerase and T4 DNA ligase, the

heteroduplex DNA is transformed into the E. coli strain MV 1190 with specifications as stated above. In each of these cases, the repair pr cess in this bacterial host will eliminate the uracils in the parenteral strands and at the same time, the in vitro synthesized strand will serve as a repair template whereby the introduced DNA changes will be conserved. All the positive clones will be DNA sequenced and the expression cassettes isolated as described above and inserted into the yeast shuttle vector YEp 24 for transformation of Saccharomyces cerevisiae.

This general approach with the specific alterations as indicated, enables the generation of any desired PTH peptide and PTH like peptide. For example, amino acid substitutions, deletions, insertions or extensions confined within the first 26 amino acids in the N-terminal region can produce agonists with increased affinity for the PTH receptors as well as antagonists which bind to the receptor, but ar biologically inactive. The mid-region or the C-terminal part of the molecule is of importance for modifying the binding of PTH to the different receptors in bone cells and the kidney. Changes in either of these regions produce an increased or diminished binding affinity to the receptors in bone cells and the kidney, and this may propose specialization in binding characteristics so that the PTH derivative could bind and function only in bone cells or in the kidney, or alteration, i.e., stimulation or blockade, of the biological activity at one or both receptor sites.

The inventions have been described herein with reference to certain preferred embodiments. However, as obvious variations thereon will become apparent to those skilled in the art, the inventions are not to be considered limited thereto.

EXAMPLE 16

Comparison of the Biological Activity of Human Parathyroid Hormone (hPTH 1-84, Bachem Fine Chemicals, Cal. USA) with QPTH

The purpose of this study was to compare the biological activity of the recombinant QPTH with the standard PTH preparation of Bachem human PTH (1-84). We examined the ability of the two agents to induce hypercalcemia in rats. Both the maximum plasma calcium levels as well as the duration of action was monitored.

Methods:

Male Wistar rats (150-200) were parathyroidectomized using electrocautery 18 hours before the start of the experiment. The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml per rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers Acetate, 4% bovine serum albumin (BSA), and 25 units heparin/ml. Five minutes after injection of 200 µl of the heparinized Ringers, a baseline blood sample was drawn (300 µl). The animals were trachesostomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH was then injected subcutaneously, in a volume of 200 µl. Both hPTH and QPTH had been dissolved into 50 µl of 0.01 N acetic acid, allowing at least one half hour for complete dissolution. After dissolving in the acetic acid, the agents were brought up in 450 µl of Ringers Acetate containing 1% BSA. Blood samples were then drawn at 1, 2, 3, and 4 hours after the injection of the PTH. The rats were reheparinized 5 minutes before drawing each blood sample using 200 µl of the heparinized Ringers solution.

The blood samples were centrifuged in a clinical centrifuge for 10 minutes, then the plasma was analyzed for calcium using a Cobas autoanalyzer.

Both the Bachem hPTH and the QPTH induced hypercalcemia in the rats to about the same degree and lasting about 2 hours. No significant difference in the calcium response was seen until 4 hours after the injections. Then the QPTH maintained the serum calcium better ($p<0.05$) than synthetic Bachem PTH.

The zero time plasma calcium (baseline) indicates the time of PTH injection and was set equal to zero. The changes in plasma calcium from zero are given as positive or negative values depending on the change (increase or reduction) in the measured values.

Time after injection (hrs)

[calcium mg/100 ml from baseline]

	Median values			
Preparation	**1**	**2**	**3**	**4 hours**
Bachem hPTH				
baseline: 6.84±0.30				
(mg/100 ml)	+0.45	+0.30	-0.20	-0.70*
QPTH				
baseline: 7.011±0.29				
(mg/100 ml) (n=7)	+0.55	+0.25	0.0	-0.50

*a significant difference of p 0.05 (Wilcoxon, two-sided test)

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